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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
B03C 1/00, G01N 33/543
C12N 11/00

A1

(11) International Publication Number: WO 91/09678

(43) International Publication Date: 11 July 1991 (11.07.91)

(21) International Application Number: PCT/US90/07492

(22) International Filing Date: 18 December 1990 (18.12.90)

(30) Priority data:

455,071	22 December 1989 (22.12.89)	US
566,169	10 August 1990 (10.08.90)	US

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(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ORGANO-METALLIC COATED PARTICLES FOR USE IN SEPARATIONS

(57) Abstract

Magnetically responsive particles and to their use in systems in which the separation of certain molecules from the surrounding medium is necessary or desirable are disclosed. The magnetically responsive particles consist of a metal, metal oxide or metal alloy core, coated with an organo-metallic polymer having attached thereto an organic functionality to which a variety of organic and/or biological molecules can be coupled. The particles can be dispersed in aqueous media without rapid gravitational settling and conveniently reclaimed from the media using a magnetic field. The magnetically responsive particles of the invention may be coupled to biological or organic molecules with affinity for, or the ability to absorb, or which interact with certain other biological or organic molecules. Particles so coupled may be used in a variety of *in vitro* or *in vivo* systems involving separations steps or the directed movement of coupled molecules to particular sites, including immunological assays, other biological assays, biochemical or enzymatic reactions, affinity chromatographic purification, cell sorting and diagnostic and therapeutic uses.

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ORGANO-METALLIC COATED PARTICLES FOR USE IN SEPARATIONS

Description

Background of the Invention

Magnetic separations in biological systems have been used as an alternative to gravitational or centrifugal separations. B.L., Hirschbein, et al., Chemtech, pp. 172-179 (1982); M. Pourfarzaneh, The Ligand Quarterly, 5(1):41-47 (1982); and P.J., and P., Dunhill, Enzyme Microb. Technol. 2:2-10 (1980). There are several advantages to using magnetically separable particles as supports for biological molecules such as enzymes, antibodies and other bioaffinity adsorbents. For example, when magnetic particles are used as solid phase supports in immobilized enzyme systems, the enzyme can be selectively recovered from the media, including media containing suspended solids, allowing recycling in enzyme reactors. P.J., Robinson, et al., Biotech. Bioeng., 15:603-606 (1973). When used as solid supports in immunoassays or other competitive binding assays, magnetic particles permit the reaction to occur under homogeneous conditions, which promotes optimal binding

kinetics, minimally alters analyte-adsorbent equilibrium and facilitates separation of bound from unbound analyte, particularly as compared to centrifugation.

Centrifugal separations are time-consuming, require expensive and energy-consuming equipment and pose radiological, biological and physical hazards. Magnetic separations are relatively rapid and easy, requiring simple equipment. The use of non-porous adsorbent-coupled magnetic particles in affinity chromatography systems allows better mass transfer and results in less fouling of the sample than in conventional affinity chromatography systems.

The practical development of magnetic separations has been hindered by several critical properties of the magnetic particles developed thus far. For example, large magnetic particles (e.g., having a mean diameter in solution greater than 10 microns) respond to weak magnetic fields and magnetic field gradients; however, they tend to settle rapidly, limiting their usefulness for reactions requiring homogeneous conditions. Large particles also have a more limited surface area per weight than smaller particles, so that less material can be coupled to them. Examples of large particles are those described by Robinson et al., in Biotech. Bioeng., 15:603-606 (1973), which are 50-125 microns in diameter; particles described by Mosbach and Anderson in Nature, 270:259-261 (1977), which are 60-140 microns in diameter and those described by Guesdon et al., in J. Allergy Clin. Immunol., 61(1):23-27 (1978) which are 50-160 microns in diameter.

Smaller magnetic particles have been described. For example, composite particles described by Hersh and Yaverbaum, in U.S. Patent No. 3,933,997, are ferromagnetic iron oxide (Fe_3O_4) carrier particles, which were reported to have diameters between 1.5 and 10 microns. However, based on the reported settling rate of 5 minutes and coupling capacity of only 12 milligrams of protein per gram of particles, the actual size of the particles in solution is expected to be substantially greater than 10 microns. L.S. Hersh and S. Yaverbaum, Clin. Chem. Acta, 63:69-72 (1975).

Small magnetic particles (e.g., having a mean diameter in solution less than about 0.03 microns) can be kept in solution by thermal agitation and do not tend to settle spontaneously. However, the magnetic field and magnetic field gradient required to remove such particles from solution are large and require heavy and bulky magnets for generating these fields, which are inconvenient to use in bench top work. Magnets capable of generating magnetic fields in excess of 5000 Oersteds, for example, are typically required to separate magnetic particles of less than 0.03 microns in diameter.

The ferromagnetic carrier particles must generally be coated in order to provide a reactive substrate for attaching chemical functional groups to bind enzymes or antibodies, for example. Silane polymers are often used for this purpose. Particles described in U.S. Patent No. 3,933,997 are coated with silanes capable of reacting with anti-digoxin antibodies to chemically couple the antibodies to the carrier particles. Various silane couplings are discussed in U.S. Patent No. 3,652,761,

which is hereby incorporated by reference. Procedures for silanization known in the art generally differ from each other in the media chosen for the polymerization of silane and its disposition on reactive surfaces. The medium is generally an organic solvent such as toluene (H.W., Weetall, In: Methods of Enzymology, K. Mosbach (ed), 44:134-148, 140 (1976)), methanol (U.S. Patent No. 3,933,997) or chloroform (U.S. Patent No. 3,652,761). Silane depositions from aqueous alcohol and aqueous solutions with acid have also been used. H.W. Weetall, In: Methods in Enzymology, supra, p.139 (1976).

There are several drawbacks to silane-coated particles. For example, the dehydration methods used to dry the coatings, such as air and/or oven drying, allow the silanized surfaces of the carrier particles to contact each other, potentially resulting in interparticle bonding, including cross-linking between particles by siloxane formation, van der Waals interactions or physical adhesion between adjacent particles. This interparticle bonding yields covalently or physically bonded aggregates of silanized carrier particles of considerably larger diameter than individual carrier particles. Such aggregates have low surface area per unit weight and hence, a low capacity for coupling with molecules such as antibodies, antigens or enzymes. Such aggregates also have gravitational settling times which are too short for many applications.

Magnetic particles capable of binding bioaffinity reagents are useful in separating desired biological components from a sample, for example, in radioimmunoassay. Radioimmunoassay (RIA) is a term used to

describe methods for analyzing the concentrations of substances involving a radioactively labeled substance which binds to an antibody. The amount of radioactivity bound is altered by the presence of an unlabeled test substance capable of binding to the same antibody. The unlabeled substance, if present, competes for binding sites with the labeled substance and thus decreases the amount of radioactivity bound to the antibody. The decrease in bound radioactivity can be correlated to the concentration of the unlabeled test substance by means of a standard curve. An essential step of RIA is the separation of bound and free label which must be accomplished in order to quantitative the bound fraction.

A variety of conventional separation approaches have been applied to RIA including coated tubes, particulate systems, and double antibody separation methods. Coated tubes, such as described in U.S. Patent No, 3.646,346 allow separation of bound and free label without centrifugation but suffer from two major disadvantages. First, the surface of the tube limits the amount of antibody that can be employed in the reaction. Second, the antibody is far removed (as much as 0.5 cm) from the antigen, slowing the reaction between the antibody and antigen. G.M. Parsons, In: Methods in Enzymology, J.F. Langone (ed), 73:225 (1981); P.N. Nayak, The Ligand Quarterly, 4(4):34 (1981).

Antibodies have been attached to particulate systems to facilitate separations. U.S. Patent Nos. 3,652,761 and 3,555,143. Such systems have large surface areas permitting nearly unlimited amounts of antibody to be used, but the particulates frequently settle during the

assay. The tube frequently must be agitated to achieve even partial homogeneity P.M. Jacobs, The Ligand Quarterly, 4(4):23-33 (1981). Centrifugation is still required to effect complete separation of bound and free label.

Antibodies may react with labeled and unlabeled molecules followed by separation using a second antibody raised to the first antibody. The technique, termed the doubled antibody method, achieves homogeneity of antibody during reaction with label but requires an incubation period for reaction of first and second antibodies followed by a centrifugation to pellet the antibodies.

Antibodies have been attached to magnetic supports in an effort to eliminate the centrifugation steps in radioimmunoassays for nortriptyline, methotrexate, digoxin, thyroxine and human placental lactogen. R.S. Kamel et al., Clin. Chem., 25(12):1997-2002 (1979); R.S. Kamel and J. Gardner, Clin. Chem. Acta., 89:363-370 (1978); U.S. Patent No., 3,933,997; C. Dawes and J. Gardener, Clin. Chem. Acts, 86:353-356 (1978); D.S. Ithakissios et al., Clin. Chem. Acta., 84:69-84 (1978); D.S. Ithakissios and D.O. Kubiatowicz, Clin. Chem., 23(11):2072-2079 (1977); and L. Nye et al., Clin. Chem. Acta., 69:387-396 (1976), the teachings of the above are hereby incorporated by reference. Such methods suffer from large particle sizes (10-100 microns in diameter) and require agitation to keep the antibody dispersed during the assay. Since substantial separation occurs from spontaneous settling in the absence of a magnetic field these previous methods are in fact only magnetically assisted gravimetric separations. Davies and

Janata in U.S. Patent No. 4,177,253 employed magnetic materials such as hollow glass or polypropylene (4-10 microns in diameter) with magnetic coatings (2 microns 10 microns thick) covering a proportion of the particle surface. Antiestradiol antibodies were coupled to such particles and their potential usefulness in estradiol RIAs was demonstrated. While this approach may have over come the problem of settling, the particle size and the magnetic coating nonetheless present limitations on surface area and hence limitations on the availability of sites for antibody coupling.

Magnetic separations have been applied in other biological systems besides RIA. Several nonisotopic immunoassays, such as fluoroimmunoassays (FIA) and enzyme-immunoassays (EIA) have been developed which employ antibody-coupled (or antigen coupled) magnetic particles. The principle of competitive binding is the same in FIA and EIA as in RIA except that fluorophores and enzymes, respectively, are submitted for radioisotopes as label. By way of illustration, M. Pourfarzaneh et al., and R.S. Kamel et al., developed magnetizable solid-phase FIA's for cortisol and phenyltoin, respectively, utilizing ferromagnetic cellulose/iron oxide particles to which antibodies were coupled by cyanogen bromide activation. M. Pourfarzaneh et al., Clin. Chem., 26(6):730-733 (1980); R.S. Kamel et al., Clin. Chem., 26(9):1281-1284 (1980).

A non-competitive solid phase sandwich technique EIA for the measurement of IgE was described by J.L. Guesdon et al., in J. Allergy Clin. Immunol., 61(1):23-27 (1978). By this method, anti-IgE antibodies coupled by

glutaraldehyde activation to magnetic polyacrylamide agarose beads are incubated with a test sample containing IgE to allow binding. Bound IgE is quantitated by adding a second anti-IgE antibody labeled with either alkaline phosphatase or β-galactosidase. The enzyme labeled second antibody complexes with IgE bound to the first antibody, forming the sandwich, and the particles are separated magnetically. Enzyme activity associated with the particles, which is proportional to bound IgE is then measured permitting IgE quantitation.

A magnetizable solid phase non-immune radioassay for vitamin B12 has been reported by D.S. Ithakissios and D.O. Kubiatowicz Clin. Chem., 23(11):2072-2079 (1977). The principle of competitive binding in non-immune radioassays is the same as in RIA with both assays employing radioisotopic labels. However, while RIA is based on the binding or interaction of certain biomolecules like vitamin B12 with specific or non-specific binding, carrier, or receptor proteins. The magnetic particles of Ithakissios and Kubiatowicz were composed of barium ferrite particles embedded in a water-insoluble protein matrix.

In addition to their use in the solid phase biological assays just described, magnetic particles have been used for a variety of other biological purposes. For example, magnetic particles have been used in cell sorting systems to isolate select viruses, bacteria and other cells from mixed populations. U.S. Patent Nos., 3,970,518; 4,230,685; and 4,267,234, the teachings of which are hereby incorporated by reference. They have been used in affinity chromatography systems to

selectively isolate and purify molecules from solution and are particularly advantageous for purification from colloidal suspensions. K. Mosbach and L. Anderson, Nature 170:259-261 (1977), hereby incorporated by reference. Magnetic particles have also been used as the solid phase support immobilized enzyme systems. Enzymes coupled to magnetic particles are contacted with substrates for a time sufficient to catalyze the biochemical reaction. Thereafter, the enzyme can be magnetically separated from products and unreacted substrate and potentially can be reused. Magnetic particles have been used as supports for α-chymotrypsin, β-galactosidase (U.S. Patent No. 4,152,210), hereby incorporated by reference) and glucose isomerase (U.S. Patent No. 4,343,901, hereby incorporated by reference) in immobilized enzyme systems.

Summary of the Invention

The present invention relates to magnetically responsive particles coated with an organo-metallic polymer capable of binding bioaffinity adsorbents, and to their use in the separation of biological molecules from, or directed movement of the molecules in, the surrounding medium. The organo-metallic coating is adsorbed onto or covalently bound to the magnetic particle. Methods and compositions for preparing and using organo-metallic coated magnetic particles are provided.

The magnetic particles comprise a magnetically responsive metal, metal alloy, or metal oxide core surrounded by an organo-metallic polymer coating which is adsorbed or covalently bound to the particle. The

organo-metallic polymer is formed from an organo-metallic monomer, which is applied to the metal particle, and thermally cross-linked in situ to form an adsorbed or a covalently bound polymer coating. Organo-titanium polymers are preferred, however, organo-metallic polymers formed from coordinate complexes of other transition metals, such as zirconium (Zr), hafnium (Hf), vanadium (V), tantalum (Ta) and niobium (Nb), or post-transition metals, such as tin (Sn) and antimony (Sb), can be used. A wide variety of bioaffinity adsorbents can be covalently bonded to the organo-metallic polymer coating through selected coupling chemistries.

More particularly, the invention relates to methods for the preparation of magnetically responsive particles comprising a metal, metal alloy or metal oxide core and an organo-metallic coating having an aliphatic moiety and an organic functionality to which a variety of organic and/or biological molecules can be coupled. The particles, coupled or uncoupled, can be dispersed in aqueous media forming a colloidal dispersion which is stable, that is, the particles resist rapid gravitational settling. The particles can be reclaimed from the media by applying a magnetic field.

Preferably, the particles are superparamagnetic; that is, they exhibit no reminent magnetization after removal of a magnetic field which allows the particles to be redispersed without magnetic aggregate formation.

The organo-metallic coated magnetically responsive particles of the invention may be coupled through the organic functionality to biological or organic molecules

with affinity for, or the ability to adsorb, or which interact with, certain other biological or organic molecules. Particles so coupled may be used in a variety of *in vitro* or *in vivo* systems involving separations steps or the directed movement of coupled molecules to particular sites, including immunological assays, other biological assays, biochemical or enzymatic reactions, affinity chromatographic purification, cell sorting and diagnostic and therapeutic uses.

The present organo-metallic coated magnetic particles provide superior composition, size, surface area, coupling versatility, settling properties and magnetic behavior for use in biological separations. The magnetic particles of this invention are suitable for many of the assays, enzyme immobilization, cell sorting and affinity chromatography procedures reported in the literature and, in fact, overcome many of the problems associated with particle settling and reuse experienced in the past with such procedures.

Detailed Description of the Invention

The magnetically responsive particles of this invention overcome problems associated with the size, surface area, gravitational settling rate and magnetic character of previously developed magnetic particles. Gravitational settling times in excess of about 24 hours can be achieved with the present magnetic particles. The gravitational settling time is defined to be the time for the turbidity of a dispersion of particles to fall by fifty percent in the absence of a magnetic field gradient.

The present magnetic particles comprise a core of a magnetically responsive metal, metal alloy or metal oxide, coated with organo-metallic polymer, which is capable of binding reactive groups or agents, for example, chemically reactive groups, biologically reactive groups or bioaffinity agents. The organo-metallic polymer is adsorbed onto or covalently bound to the magnetic particle. The term "magnetically responsive particle" or "magnetic particle" is defined as any particle dispersible or suspendible in aqueous media without significant gravitational settling, and separable from suspension by application of a magnetic field.

The term "magnetic core" is defined as a crystal or group (or cluster) of crystals of a transition metal, alloy or magnetic metal oxide having ferrospinel structure and comprising trivalent and divalent cations of the same or different transition metals or magnetic metal crystal group. Metals, alloys and oxides which are useful as magnetic core material in the present invention include the metals, alloys and oxides based on metals which appear in the Periodic Table in Groups 4a and b, 5a and b, 6a and 7a. These include, for example, divalent transition metals, such as iron, magnesium, manganese, cobalt, nickel, zinc and copper, alloys of these metals such as iron alloys or oxides (e.g., iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide), cobalt ferrite, samarium cobalt, barium ferrite, and aluminum-nickel-cobalt and metal oxides including magnetite (Fe_3O_4), hematite (Fe_2O_3) and chromium dioxide (CrO_2). By way of illustration, a magnetic core may be

comprised of a cluster of superparamagnetic crystals of iron oxide, or a cluster of superparamagnetic or ferromagnetic crystals of irons or oxide, or may consist of an single superparamagnetic or ferromagnetic crystal of an iron oxide or metal alloy.

The present particles are preferably between about 0.003 and about 1.5 microns in diameter, and have a surface area of from about 50 to 150 meters/gm, which provides a high capacity for coupling of a bioaffinity adsorbent, chemical or biochemical reactive group. Magnetic particles of this size range overcome the rapid settling problems of larger particles, but obviate the need for large magnets to generate the magnetic fields and magnetic field gradients required to separate smaller particles. For example, magnets used to effect separations of the magnetic particles of this invention need only generate magnetic fields between about 100 and about 1000 Oersteds. Such fields can be obtained with permanent magnets which are smaller than the container which holds the dispersion of magnetic particles and, thus, are suitable for benchtop use.

Particles with superparamagnetic behavior are preferred since superparamagnetic particles do not exhibit the magnetic aggregation associated with ferromagnetic particles and permit redispersion and reuse. The term "superparamagnetism" is defined as that magnetic behavior exhibited by iron, cobalt, nickel or other metal alloys or metal oxides having a crystal size of less than about 300A, which behavior is characterized by

responsiveness to a magnetic field without reminant magnetization.

Ferromagnetic particles may be useful in certain applications of the invention. The term "ferromagnetism" is defined as that magnetic behavior exhibited by iron, iron alloys or iron oxides with a crystal size greater than about 500A, which behavior is characterized by responsiveness to a magnetic field with a reminant magnetization of greater than about 10 gauss upon removal of the magnetic field.

The particles or crystals are then coated with an organo-metallic monomer material capable of adsorptive or covalently bonding to the magnetic particles. Organo-metallic monomers useful for the present coated particles are organic coordinate complexes of selected transition and/or post transition metals which are capable of forming a stable coordination coumpound, and organic ligands, which can be adsorbed onto or covalently bound to the magnetic particle and, crosslinked <u>in situ</u> on the particle surface, thereby forming the organo-metallic polymer coating. The organo-metallic monomer must be able to be functionalized or derivatized in a manner that allows the polymer formed therefrom to form covalent bonds with bioaffinity or chemical affinity adsorbents. For this purpose, the organo-metallic polymer is post-functionalized or derivitized with an aliphatic "spacer arm" which is terminated with an organic functional group capable of coupling with bioaffinity adsorbents. The "spacer arm" is an aliphatic hydrocarbon having from about 2 to about 60 atoms, e.g., carbon, nitrogen and/or oxygen atoms. The purpose of the spacer arm is to

provide a non-reactive linker (or spacer) between the organic group which reacts with the chemical group, biochemical group or bioaffinity adsorbent and the polymer chain, and to impart an appropriate degree of hydrophilic/hydrophobic balance to the surface of the coated particle. The organic group is generally a reactive group such as an amine (NH_2), carboxyl group (COOH), cyanate (CN), phosphate (PO_3H), sulfate (SO_3H), thiol (SH), hydroxyl (OH) group, vinyl (C=C), nitrate (NO_2), aldehyde, epoxide, succinamide or anhydride group coupled to an aliphatic or aromatic moiety.

Particularly useful organo-metallic compounds are coordinate complexes formed from selected transition metals (e.g., Ti, Zr, Hf, V, Zn, Cd, Mn, Te, Re, Ta, Nb) and/or post-transition metals (e.g., Sn, Sb, Al, Ga, In, Ge). Organo-titanium compounds are particularly preferred. Organo-titanium compounds which are useful including, for example, titanium-tetra-isopropoxide, amino-hexyl-titanium-tri-isopropoxide, amino-propyl-titanium-tri-isopropoxide and carboxyl-hexyl-titanium-tri-isopropoxide. In one embodiment of the present invention, amino-hexyl-titanium-tri-isoproxide is coated onto the magnetic particle of choice, and thermally crosslinked to form an organo-titanium polymer coating having an aliphatic spacer arm (the hexyl moiety) and organic functional group (the amine group).

The coated particle is post-functionalized, if necessary, in a manner that allows the organo-metallic polymer to form covalent bonds with bioaffinity or chemical affinity adsorbents. In one embodiment of the present method, an organo-titanium polymer, such as

titanium-tetra-isopropoxide which lacks the spacer arm and organic functional group, is coated onto the magnetic particle of choice and partly crosslinked at about 40°C for a period of time sufficient to allow the organotitanium polymer to become adsorbed onto the particle surface. The organotitanium coated magnetic particle is then activated by reaction with an agent such as 1-hydroxy-6-amino hexane, to form the amino-hexyl-titanium-tri-isopropoxide. The coating is then crosslinked at elevated temperatures to form an organotitanium polymer coating having an aliphatic spacer arm and an organic functionality (i.e., the amine group). The functionalized particle can then be reacted or coupled, with the bioaffinity adsorbent of choice.

The magnetic core particles are prepared according to the following general procedure: metal salts are precipitated in a base to form fine magnetic metal oxide crystals. The crystals are redispersed, then washed in water and in an electrolyte. Magnetic separation can be used to collect the crystals between washes if the crystals are superparamagnetic.

In one embodiment of the present invention, superparamagnetic iron oxide particles are made by precipitation of divalent (Fe^{2+}) and trivalent (Fe^{3+}) iron salts, for example, ferrous ammonium sulfate, $Fe_2(NH_2)(SO_4)$ and ferric sulfate, $Fe_2(SO_4)_3$, in aqueous base. The ratio of Fe^{2+} and Fe^{3+} and counterion can be varied without substantial changes in the final product by increasing the amount of Fe^{2+} while maintaining a constant molar amount of iron. Counterions including nitrate, sulfate, chloride or hydroxide are useful in the method. A

Fe^{2+}/Fe^{3+} ratio of about 2:1 to about 4:1 is useful in the present invention; a ratio of about 2:1 $Fe^{2+}:Fe^{3+}$ is particularly useful. An Fe^{2+}/Fe^{3+} ratio of 1:1 produces magnetic particles of slightly inferior quality to those resulting from the higher Fe^{2+}/Fe^{3+} ratios, the particle size is more heterogeneous than that resulting from Fe^{3+}/Fe^{2+} of 2:1 or 4:1.

In this embodiment, aqueous solutions of the iron salts are mixed in a base, such as ammonium, sodium or potassium hydroxide, which results in the formation of a crystalline precipitate of superparamagnetic iron oxide. The precipitate is washed repeatedly with water by magnetically separating and redispersing it until a neutral pH is reached. The precipitate is then washed with about five equal portions of a water miscible solvent, such as acetone, methanol or ethanol that has been dried over molecular sieves to remove all of the water.

The repeated use of magnetic fields to separate the iron oxide from suspension during the washing steps is facilitated by the superparamagnetic properties of the crystals. Regardless of how many times the particles are subjected to magnetic fields, they never become magnetically agglomerated and consequently, can be redispersed by mild agitation. Ferromagnetic particles cannot be prepared by this washing procedure as they tend to magnetically aggregate after exposure to magnetic fields and cannot be homogeneously redispersed.

Other divalent transition metal salts such as magnesium, manganese, cobalt, nickel, zinc and copper salts may be substituted for iron salts in the

precipitation or milling procedure to yield magnetic metals or metal oxides. For example, the substitution of divalent cobalt chloride ($CoCl_2$) for $FeCl_2$ in the above procedure produced ferromagnetic metal oxide particles. Ferromagnetic metal oxide particles such as those produced with $CoCl_2$ can be washed in the absence of magnetic fields by employing conventional techniques of centrifugation or filtration between washings to avoid magnetizing the particles. As long as the resulting ferromagnetic metal oxides are of sufficiently small diameter to remain dispersed in aqueous media, they can also be coated with the organo-metallic polymer and coupled to bioaffinity adsorbents for use in systems requiring a single magnetic separation, e.g., certain radioimmunoassays. Ferromagnetism limits particle usefulness in those applications requiring redispersion or reuse.

In another embodiment of the present invention, the magnetic core particles can be made by precipitating metal powders and reducing the particle size by milling the resulting precipitate, for example, in a ball mill. In this process, the metal powder is precipitated from an aqueous solution of, for example, Fe^{+2} or Fe^{+3} salt with sodium borohydride. For example, an aqueous solution of ferrous chloride ($FeCl_2$) is mixed with sodium borohydride ($NaBH_4$) to form a fine iron precipitate. The resulting properties of the metal powder are unaffected by the valance of the counter ion or iron metal salt selected. Complete precipitation occurs spontaneously upon borohydride addition. The magnetic metal powder is then collected by filtration and washed with about five equal

volumes of water to remove all soluble salts, then washed with five equal volumes of dry in acetone to remove all residual water. The particle is added as an aqueous slurry in a concentration of about 1-25% to a commercial ball mill filled half way with 1/4" stainless steel balls and milled for 3-30 days. At the completion of the milling period, a superparamagnetic metal slurry is formed and coated and functionalized as the superparamagnetic particles described in the previous section.

In another embodiment of the present invention, the magnetic core particles are made by reacting a metallocene, e.g., particulate ferrocene (dicyclopentadenyliron, $C_{10}H_{10}Fe$) with iron (II) hydroxide. In this embodiment, an aqueous ferrocene (or other metallocene) slurry is prepared, and an aqueous slurry of iron (II) hydroxide is prepared separately. The ferrocene slurry is prepared, for example, by milling a mixture of ferrocene and water in a ball mill. The iron (II) hydroxide slurry can be prepared, for example, by precipitating an aqueous solution of ferrous sulfate with ammonium hydroxide to form ferrous hydroxide. The two slurries are then combined and milled, for example, forming fine magnetite particles. Other metallocene compounds (e.g., nickelocene, cobaltocene) can be mixed with the ferrocene to produce various magnetic ferrite particles. This process is described in detail in co-pending U.S. patent application Serial No. __________, [Attorney's Docket No. OQC90-02] by M.S. Chagnon, filed concurrently herewith, the teachings of which are hereby incorporated herein by reference.

In one embodiment of the present invention, the coating around the magnetic core particle is amino-propyl-titanium-tri-isopropoxide. The polymerization is performed by redispersing the magnetic particle in an acetone solution, adding the organo-titanium monomer, then crosslinking with heat. The terms "coupled magnetically responsive particle" or "coupled magnetic particle" refer to any magnetic particle to which one or more types of bioaffinity adsorbents are coupled by covalent bonds, which covalent bonds may be amide, ester, ether sulfonamide, disulfide, azo or other suitable organic linkages depending on the functionalities available for bonding on both the coating of the magnetic particle and the bioaffinity adsorbents.

Preferred magnetically responsive particles of the present invention have metal oxide cores composed of clusters of superparamagnetic crystals affording efficient separation of the particles in low magnetic fields (100-1000 Oersteads) while maintaining superparamagnetic properties. Aggregation of particles is controlled during particle synthesis to produce particles which are preferably small enough to avoid substantial gravitational settling over times sufficient to permit dispersions of the particles to be used in an intended biological assay or other application. The advantage of having superparamagnetic cores in magnetically responsive particles is that such particles can be repeatedly exposed to magnetic fields. Superparamagnetic particles do not exhibit reminent magnetization and have no coercive strength, and, therefore, do not magnetically aggregate, thus, the particles can be redispersed and

reused. Even after coating, preferred particles of the invention having cores made up of clusters of crystals exhibit a remarkably high surface area per unit weight and a generally corresponding high coupling capacity, which indicates that such particles have an open or porous structure.

The bioaffinity adsorbents can be covalently bonded to the organo-metallic coated magnetic particles of this invention by conventional coupling chemistries. Several coupling reactions can be performed. For example:

(a) If the ligand to be coupled contains an amino group, it can be coupled directly to the activated organo-metallic polymer. If a different functionality is desired, it can be introduced, for example, by adding a spacer arm containing the functionality by sequential reaction of the organo-metallic polymer (e.g., titanium-tetra-isopropoxide) with any omega-functional higher molecular weight alcohol. The amino group on the ligand can then be coupled to the free functional group on the spacer arm; or

(b) If the ligand contains an aldehyde group instead of an amino group, it can be coupled directly to the free amino group of an amino alkane (that is, an alkane spacer arm having an amino functionality) on the coated magnetic particle.

The term "bioaffinity adsorbent" is defined as any biological or other organic molecule capable of specific or nonspecific binding or interaction with another biological molecule, which binding or interaction may be referred to as "ligand/ligate" binding or interaction and is exemplified by, but not limited to, antibody/antigen,

antibody/hapten, enzyme/substrate, carrier protein/substrate, lectin/carbohydrate, receptor/hormone, receptor/effector or repressor/inducer bindings or interactions.

The coupled organo-metallic coated magnetic particles of the present invention can be used in immunoassays or other binding assays for the measurement of analytes in solution. The term "immunoassay" is defined as any method for measuring the concentration or amount of an analyte in a solution based on the immunological binding or interaction of a polyclonal or monoclonal antibody and an antigen, which method (a) requires a separation of bound from unbound analyte; (b) employs a radioisotopic, fluorometric, enzymatic, chemiluminescent or other label as the means for measuring the bound and/or unbound analyte; and (c) may be described as "competitive" if the amount of bound measurable label is generally inversely proportional to the amount of analyte originally in solution or "non-competitive" if the amount of bound measurable label is generally directly proportional to the amount of analyte originally in the solution. Label may be in the antigen, the antibody, or in double antibody methods, the second antibody. Immunoassays are exemplified by, but are not limited to, radioimmunoassays (RIA), immunoradiometric assays (IRMA), fluoroimmunoassays (FIA), enzyme immunoassays (EIA), and sandwich method immunoassays. The analyte or the bioaffinity adsorbent can include, for example, antibodies, antigens, haptens, enzymes, apoenzymes, enzymatic substrates, enzymatic inhibitors, cofactors, nucleic acids, binding proteins, carrier proteins, compounds bound by binding proteins, compounds bound by carrier

proteins, lectins, monosaccharides, polysaccharides, hormones, receptors, repressors and inducers.

Such assays are preferably carried out by mixing a sample containing an unknown concentration of analyte with a known amount of labeled analyte in the presence of magnetic particles coupled to a bioaffinity adsorbent capable of binding to, or interacting with, both unlabeled and labeled analyte, allowing the binding or interaction to occur, magnetically separating the particles, measuring the amount of label associated with the magnetic particles and comparing the amount of label to a standard curve to determine the concentration of analyte in the sample.

The term "binding assay" or "non-immune assay" is defined as any method for measuring the concentration or amount of an analyte in solution based on the specific or nonspecific binding or interaction, other than antibody/antigen binding or interaction, or a bioaffinity adsorbent and another biological or organic molecule, which method (a) requires a separation of bound from unbound analyte; (b) employs a radioisotopic, fluorometric, enzymatic, chemiluminescent or other label as as the means for measuring the bound and/or unbound analyte; and (c) may be described as "competitive" if the amount of bound measurable label is generally inversely proportional to the amount of analyte originally in solution or "non-competitive" if the amount of bound measurable label is generally originally in solution.

The magnetic organo-metallic-coated particles of this invention are useful in immobilized enzyme systems, particularly where enzyme recycling is desired. The term

"immobilized enzyme system" is defined as any enzymatically catalyzed biochemical conversion or synthesis or degradation wherein the enzyme molecule or active site thereof is not freely soluble but is adsorptively or covalently bound to a solid phase support, which support is suspended in or contacted with the surrounding medium and which may be reclaimed or separated from said method. In this embodiment, enzymatic reactions are carried out by dispersing enzyme-coupled magnetic particles in a reaction mixture containing one or more substrates, under conditions sufficient for the reaction between the enzyme and substrate to occur, magnetically separating the enzyme-magnetic particle from the reaction mixture containing products and unreacted substrates and, if desired, redispersing the particles in fresh substrates thereby reusing the enzyme.

Affinity chromatography separations and cell sorting can be performed using the magnetic particles of this invention. The term "affinity chromatography" is defined as a method for separating, isolating, and/or purifying a selected molecule from its surrounding medium on the basis of its binding or interaction with a bioaffinity adsorbent adsorptively or covalently bound to a solid phase support, which support is suspended in or contacted with the surrounding medium and which may be reclaimed or separated from said medium by dispersing bioaffinity adsorbent coupled magnetic particles in solutions or suspensions containing molecules or cells to be isolated and/or purified, allowing the bioaffinity adsorbent and the desired molecules or cells to interact, magnetically separating the particles from the solutions or suspension

and recovering the isolated molecules or cells from the magnetic particles.

It is further contemplated that the organo-metallic coated magnetic particles of this invention can be used in *in vivo* systems for the diagnostic localization of cells or tissues recognized by the particular bioaffinity adsorbent coupled to the particle and also for magnetically directed delivery of therapeutic agents coupled to the particles to pathological sites.

Magnetic separation times of less than about ten minutes can be achieved with magnetic particles of the invention by contacting a vessel containing a dispersion of the particles with a pole face of a permanent magnet no larger in volume than the volume of the vessel. Magnetic separation time is defined to be the time for the turbidity of the dispersion to fall by 95 percent.

Furthermore, the use of functionalized organo-metallic polymers as the coating surrounding the metal oxide core of the magnetic particles described herein make possible the coupling of a wide variety of molecules under an equally wide variety of coupling conditions compared to other magnetic particle coatings known in the art with more limited coupling functionalities.

The invention is further illustrated by the following Examples.

EXAMPLES

Example 1: Preparation of Superparamagnetic Magnetite Particles

200 grams (1.58 moles) of ferrous chloride (VWR Scientific) and 325 grams (2.0 moles) of ferric chloride

were dissolved in 3 liters of water. 2000 grams of ammonium hydroxide (VWR Scientific) concentrate were added at a rate of 50 ml/minute under constant agitation, during which time the temperature of the solution was kept between 25 and 40°C. After the addition of the ammonium hydroxide was complete, the magnetic particle (Fe_3O_4) aqueous slurry was allowed to cool to room temperature.

Example 2: Preparation of Amino-Hexyl-Titanium-Tri-Isopropoxide

0.1 moles of titanium-tri-isopropoxide (Tyzor TPT Dupont, Wilmington, DE) and 0.1 moles of 6-amino-1-hexanol were added to a 50 ml beaker and stirred at room temperature for 1 minute to form 0.1 mole of amino-hexyl-titanium-tri-isopropoxide. The reaction mixture was heated to 70°C for 10 minutes to evaporate the isopropyl alcohol formed during the reaction.

The material was cooled to room temperature and used as a monomer in making the tetravalent titanium organo-metallic coating in Example #3.

Example 3: Preparation of Amine Functional Organo-titanate Coated Magnetic Particle

According to the procedure set out in Example 1, 4 moles $FeCl_3$ and 2 moles of $FeCl_2$ were dissolved in 4 L of distilled water and precipitated with 16 moles of ammonium hydroxide. The precipitate was washed 5 times with water and 3 times with acetone. N,N-dimethyl formamide (DMF) was added to the precipitate in the following ratio: 10 ml of DMF per gram of Fe_3O_4. The mixture was loaded into a Eiger Mill and milled

continuously for 10 minutes. The mixture was then transferred to a beaker and heated with stirring for 30 minutes at 100°C. The amine functional organo-titanate prepared in Example 2 was immediately added after preparation with constant stirring to the mixture in a ratio of 1 g dry Fe_3O_4 per 3 g of amine functional organo-titanate.

This mixture was then heated with stirring for 20 minutes at 65°C and then passed through the Eiger Mill for two passes. The resulting material was washed five times with water, the coated particles were collected with an external magnetic field of 2000 gauss and the aqueous waste was decanted.

Example 4: Preparation of An Alternating Functional-Non Functional Organo-Titanate Monomer

The procedure described in Example 2 was followed except that the organo-titanate was reacted with a comixture of amino-functional hexanol and hexanol to produce a monomer having reduced amine functionality. Hexanol and 6-amino-1-hexanol in a molar ratio of 6:1 were mixed in a 50 ml beaker for one minute. Tyzor TPT was added to the alcohol mixture in the ratio of 1 mole of alcohol per mole of Tyz or TPT. The reaction mixture was stirred for one minute, heated to 70°C for 10 minutes to evaporate the isopropyl alcohol produced by the reaction and cooled to room temperature. The resulting compound was an organotitanate, 6-amino-hexyl-titanium-tri-isopropoxide having alternating non-functional hexyl groups, that is, hexyl chains lacking the amino group. The weight ratios of 6-amino-1-hexanol:Tyzor TPT:hexanol

were 1:26:9.6. This compound was used as a monomer to make an organo-titanium coating as described in Example 5.

Example 5: Preparation of Amine Functional Organo-titanate Magnetic Particles

The procedure described in Example 3 was followed except that the amine-functional organo-titanate was the material prepared in Example 5. The mixture of magnetic particles and organo-titanate monomer was heated to 95°C for one hour with constant stirring and milled in an Eiger Mill for 4 minutes. The mixture was washed nine times with water. Adipic acid was added in the ratio of 0.5 moles of adipic acid per mole of total particles. One mole of carbodiimide (CDI) was added, and the mixture was mixed for 30 minutes on a ball mill. 1,6 hexane-diamine was added in the ratio of 0.5 moles of 1,6 hexane-diamine per mole of total particles. One mole of CDI was added and the mixture was mixed for 30 minutes. The resulting material was washed five times with water, the particles were collected using an external magnetic field of 2000 gauss and the aqueous waste was decanted.

Example 6: Preparation of Subdomain Magnetite Particles by Reaction of Particulate Ferrocene and Iron(II) Hydroxide

A 100 g of a slurry containing 20% ferrocene (by weight) (dicyclopentadenyliron; Strem Chemical Co., Newburyport, MA) in water was prepared by mixing the ferrocene with the water. The slurry was added to a commercial ball mill. The mill was filled halfway with ¼" stainless steel balls and the slurry was milled for a

period of 2 hours.

A second ferrous hydroxide slurry (iron (II) hydroxide) was made according to the following procedure. An aqueous solution containing 20g of ferrous sulfate (VWR Scientific) was precipitated using 50g of ammonium hydroxide concentrate to form gelatinous ferrous hydroxide. The gel was filtered and the filtrate washed with 5 to 100g volumes of water. The washed gel was then made into a 10% aqueous slurry and milled as previously described for 5 hours.

The ferrocene and hydroxide slurries were mixed, and the mixture was milled for one day to form fine Fe_3O_4 particles. The particles were about 100 A in diameter and were responsive to a magnetic field. These particles can be coated as described in Examples 2-5 above.

Example 7: Preparation of Subdomain Nickel-Ferrite Particles

Subdomain nickel-ferrite particles were prepared according to the procedure set out in Example 6, except that a mixture of 50g a 20% nickelocene slurry (dicyclopentadenylnickel; Strem Chemical Co., Newburyport, MA) and 50g of a 20% ferrocene slurry were used in lieu of the 100g of the ferrocene slurry in Example 6. Magnetically responsive nickel-ferrite particles having a particle size of about 100 A were produced by this method.

Example 8: Preparation Subdomain Cobalt-Ferrite Particles

Subdomain cobalt-ferrite particles were prepared according to the procedure set out in Example 6, except that a mixture of 50g of a 20% (by wt.) cobaltocene

slurry (dicyclopentadenylcobalt; Strem Chemical Co., Newburyport, MA) and 50g of the ferrocene slurry were used in lieu of 100g of the ferrocene slurry in Example 6. Magnetically responsive cobalt-ferrite particles having a particle size of about 100 A were produced by this method.

Example 9: Preparation of Subdomain Metal Particles by Sodium Borohydride Reduction and Size Reduction by Milling

200 gm (1.58 moles) of ferrous chloride was dissolved in 1 liter of water. 500 gm of dry sodium borohydride were added to the solution to form a fine iron powder precipitate. The precipitate was washed with water and collected by filtration. The filtered powder was resuspended in water and re-filtered. The washing procedure was done 4 additional times. On the final suspension, the slurry was adjusted to a concentrate of 20% and milled as described in Example 6 for a period of 75 days to produce particles with a mean diameter of less than 50 A.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, by no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following Claims.

CLAIMS

1. A coated magnetically responsive particle comprising:
   a) a magnetic core particle comprising a magnetically- responsive metal, metal alloy or metal oxide; and
   b) an organo-metallic polymer coating covalently bonded to or adsorbed onto said particle, wherein the organo-metallic polymer coating is capable of binding at least one type of bioaffinity adsorbent.

2. A coated magnetically responsive particle of Claim 1, wherein the magnetic core particle comprises a metal, metal alloy or metal oxide selected from the group consisting of iron, magnetite, iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide.

3. A coated magnetically responsive particle of Claim 2 wherein the magnetic core particle is magnetite.

4. A coated magnetically responsive particle of Claim 1 having a particle size of from about 0.003 to about 1.5 microns in diameter.

5. A coated magnetically responsive particle of Claim 1 wherein the organo-metallic polymer is formed from monomers which are coordinate complexes of organic ligands and a metal selected from the group

consisting of: titanium, zirconium, hafnium, vanadium, tanatalum, niobium, tin and antimony.

6. A coated magnetically responsive particle of Claim 5 wherein the metal is titanium.

7. A coated magnetically responsive particle of Claim 1 wherein the organo-metallic polymer is an organo-titanium polymer selected from the group consisting of: titanium-tetra-isopropoxide, amino-hexyl-titanium-triisopropoxide, amino-propyl-titanium-triisopropoxide and carboxyl-hexyl-titanium triisopropoxide.

8. A coated magnetically responsive particle of Claim 7 wherein the organo-titanium polymer is amino-hexyl-titanium-tri-isopropoxide.

9 A coated magnetically responsive particle of Claim 1 which is superparamagnetic.

10. A coated magnetically responsive particle comprising:

a) a magnetic core particle comprising a magnetically responsive metal, metal alloy or metal oxide;

b) an organo-titanium polymer coating which is covalently bonded to or adsorbed onto the particle, said organo-titanium polymer having organic functional groups attached thereto; and

c) a bioaffinity adsorbent covalently coupled to the organic function groups of the polymer coating.

11. A coated magnetically responsive particle of Claim 10, wherein the magnetic core particle is metal, metal alloy or metal oxide selected from the group consisting of: iron, magnetite, iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide.

12. A coated magnetically responsive particle of Claim 11 wherein the magnetic core comprises magnetite.

13. A coated magnetically responsive particle of Claim 10 wherein the organo-titanium polymer coating is amino-hexyl-titanium-tri-isopropoxide.

14. A coated magnetically responsive particle of Claim 10 wherein the bioaffinity adsorbent is selected from the group consisting of: antibodies, antigens, enzymes and specific binding proteins.

15. A coated magnetically responsive particle of Claim 10 which is superparamagnetic.

16. A coated magnetically responsive particle of Claim 10, wherein the organic functional groups are selected from the group consisting of: amino, carboxyl, hydroxyl, sulfate, phosphate, cyanate and thiol groups.

17. A coated magnetically responsive particle of Claim 10 having a mean diameter of from about 0.003 to about 1.5 microns.

18. A magnetically responsive particle comprising a superparamagnetic metal oxide core surrounded by an organo-titanium polymer to which bioaffinity adsorbents can be covalently coupled, the metal oxide core comprising a group of crystals of metal oxide, and the particle having a mean diameter of about 0.003 to about 1.5 microns.

19. A method of measuring analytes in a sample comprising the steps of:
   a. contacting a sample containing an unknown concentration of the analyte with a known amount of a labeled analyte in the presence of magnetic particles comprising:
      (i) a magnetic core particle comprising a magnetically responsive metal, metal alloy or metal oxide; and
      (ii) an organo-metallic polymer coating covalently bonded to or adsorbed onto said particle, wherein said organo-metallic coating having a bioaffinity adsorbent covalently coupled thereto, said bioaffinity adsorbent is capable of binding to or interacting with both the unlabeled and the labeled analyte;
   b. maintaining the mixture obtained in step (a) under conditions sufficient for said binding or interaction to occur;
   c. magnetically separating the magnetic particles; and
   d. measuring the amount of label associated with the magnetic particles and determining the concentration of analyte in the sample.

20. A method of Claim 19 wherein the analyte is selected from the group consisting of: antibodies, antigens, haptens, enzymes, apoenzymes, enzymatic substrates, enzymatic inhibitors, cofactors, nucleic acids, binding proteins, carrier proteins, compounds bound by binding proteins, compounds bound by carrier proteins, lectins, monosaccharides, polysaccharides, hormones, receptors, repressors and inducers.

21. A method of Claim 19 wherein the magnetic core particle comprises a metal, metal alloy or metal oxide selected from the group consisting of: iron, magnetite, iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide.

22. A method of Claim 21 wherein the magnetic core particle has a particle size of from about 0.003 to about 1.5 microns in diameter.

23. A method of Claim 19 wherein the organo-metallic polymer coating is formed from monomers which are coordinate complexes of organic ligands and a metal selected from the group consisting of: titanium, zirconium, hafnium, vanadium, tantalum, niobium, tin and antimony.

24. A method of Claim 23 wherein the organo-metallic polymer is an organo-titanium polymer selected from the group consisting of: titanium-tetra-isopropoxide, amino-hexyl-titanium triisopropoxide, amino-propyl-titanium isopropoxide and carboxyl-hexyl-titanium triisopropoxide.

25. A method of Claim 19 wherein the magnetically responsive particle is superparamagnetic.

26. A method of Claim 19 wherein the bioaffinity adsorbent is selected from the group consisting of: antibodies, antigens, haptens, enzymes, apoenzymes, enzymatic substrates, enzymatic inhibitors, cofactors, nucleic acids, binding proteins, carrier proteins, compounds bound by binding proteins, compounds bound by carrier proteins, lectins, monosaccharides, polysaccharides, hormones, receptors, repressors and inducers.

27. A method of Claim 19 wherein the labeled analyte is marked with a label selected from the group consisting of: radioisotopes, fluorescent compounds, enzymes and chemiluminescent compounds.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/07492

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: B 03 C 1/00, G 01 N 33/543, C 12 N 11/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC[5]	G 01 N, B 03 C

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A, 0125995 (ADVANCED MAGNETICS, INC.) 21 November 1984 see the whole document --	1-4,9,19-22, 25-27
X	EP, A, 0321322 (RHONE-POULENC CHIMIE) 21 June 1989 see the whole document --	1-4,9
A	EP, A, 0180384 (TECHNICON INSTRUMENTS CORP.) 7 May 1986 see the whole document --	1-27
A	EP, A, 0260098 (BELLEX CORP.) 16 March 1988 see the whole document --	1-27
A	EP, A, 0075346 (OCE-NEDERLAND B.V.) 30 March 1983 --	
	./.	

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th April 1991	14.06.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS M. Peis

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Chemical Abstracts, vol. 89, no. 26, 25 December 1978, (Columbus, Ohio, US), see page 656, abstract 225205h, & JP, A, 7890151 (HITACHI MAXELL, LTD) 8 August 1978 --	
A	Chemical Abstracts, vol. 108, no. 10, 7 March 1988, (Columbus, Ohio, US), see page 318, abstract 80319x, & JP, A, 62247004 (HITACHI METALS, LTD) 28 October 1987 ----------	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9007492
SA 44073

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 10/06/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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		US-A- 4443527	17-04-84

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82